HISTOLOGICAL STUDY OF THE LOWER LIMB IN WHICH MULTIPLE MILIARY EMBOLISMS WERE PRODUCED EXPERIMENTALLY : A CONTRIBUTION TO THE ALLERGIC ETIOLOGY OF THROMBOANGIITIS OBLITERANS.

AUTHOR(S):

Konishi, Seizo

HISTOLOGICAL STUDY OF THE LOWER LIMB IN WHICH MULTIPLE MILIARY EMBOLISMS WERE PRODUCED EXPERIMENTALLY. A CONTRIBUTION TO THE ALLERGIC ETIOLOGY OF THROMBOANGIITIS OBLITERANS.

From the Ist Surgical Division, Kyoto University Medical School
(Director : Prof. Dr. CHISATO ARAKI)

by

SEIZO KONISHI

(Received for Publication : Nov. 4. 1953)

INTRODUCTION

There have been many opinions about the etiology of thromboangiitis obliterans. 1) Bürger stated that the cause was inflamation of blood vessels due to a certain bacterial infection. Other authors expressed other views : 2) The pathological vasoconstriction of long duration results in the organic obstruction of the blood vessels (Schlesinger), 3) Some poisions, such as nicotine (Maddock & Coller 1933) or ergot (Kaunitz & Gerlach 1933), are the cause of dysfunction of blood vessels, 4) frost-bite or coldness is an important factor (Bier and Gruber etc.), 5) the disease is concerned with a general infectious disease (Wilhelm and Rieder etc), 6) with the increase of blood viscosity (Kaga and Maesima), 7) with some disorder of internal secretion (pituitrin-Holsclaw and Booth 1925, ovary-Boecke 1927, thyroid gland-Bassai e Digliotti, Kallos and Nusset 1933). And recently it has been assumed, 8) that important is the deficient chemical regulation of the general circulation in a pathologically sensitive organism (Ratschow). 9) There are authors who try to explain the disease from the viewpoint of allergy—Sulzberger (tobacco-extract) and Denecke (coldness, nicotine and chronic infections). Pathological changes are found in the disease not only in arteries of extremities but also arteries of nearly all organs, namely, heart, brain, mesentery, lung, kidney, spleen, adrenal gland, spermatochorda, seminal vesicle, aorta and prostate etc. Thus the allergy is regarded as a predominant etiologic factor. In addition histological changes characteristic of allergy were described in this disease by E. Jäger and recently by Wada of our laboratory. In these studies, it was assumed that the allergic changes in this disease was primary, i.e. the allergy is the primary cause of the disease. But it is possible that the allergic changes may be secondary, i.e. the devitalization of tissue resulting from the disturbed circulation may act as an allergenic agent, thus causing secondary tissue reaction of allergy.

The present work has been carried out for the elucidation of this problem.

METHODS OF EXPERIMENT

It is difficult to reproduce experimentally the progressive disturbance of arterial

blood circulation similar to that in ca e of thromboangiits obliterans, because any measure to obstruct a large artery, such as a ligature, results in the gradual recovery of disturbed circulationd ue to the collateral ve sel formation. It is desirable at least to produce a circulatory disturbance which is not readily compensated. Thus I have attempted to obturate small arteries as many as possi ble by the repeated intraarterial injection of a suspension of embolizing particles. For this purpose licopodium and silver particles were used.

Licopodiums are almost similar in size, each measuring 30-35 microns in diameter, and the form being spherical. 0.5 g. of licopodiums were suspended in 10 cc. of physiological saline solution and for about 20 minutes boiled for disinfection and at the time of injection shaken thoroughly in order to make the suspension homogeneous. The suspension contained 1060 particles of licopodium in 1 cmm. (counted in blood count chambers).

In one series of experiment, the suspension was injected alone, and in another in a mixture with hemolytic streptococcus.

The hemolytic streptococcus was cultivated at first in blood-agar and then in a bouillon for 24 hours. The fluid medium was added to the same quantity of licopodium suspension mentioned above and used.

Silver particles were prepared by Mr. Ogawa (Chemical Division, Faculty of Science, Kyoto University). Each silver particle is spherical and 20-40 microns in diameter. Particles uniformly 30-35 microns were desired, as in the case of licopodium, but not easily prepared owing to the techinical difficulty. 0.5g. of the silver particles was put into 10cc. of 2% arabic gum solution. After boiling for 20 minutes in a water bath, the mixture was brought into a suspending condition by shaking and injected rapidly, taking much care not to produce precipitation at thebottom of the injection syringe.

As experimental animals rabbits we e used. The femoral artery was exposed 1 cm. peripheral from the hip-joint. Every injection was done w th a ½mm. injection needle. The bleeding after pulling out the needle was easily arrested by pressing with gauze for a few minutes.

In each experiment, injection was repeated every 10 days. Rabbits were killed in various periods after the last injection and pieces of tissue were taken as many as possible from various parts of the injected lower limb immediately after death. The pieces were fixed in a 10 % solution of formaldehyde.

In cases of silver particle injection, the X-ray photography was done of the injected lower limb and tissue pieces were taken from the parts in which the most silver particles were visible in the picture.

For embedding paraffin was used, and in case of necessity serial sections were made. The preparations were stained with hematoxylin-eosin.

EXPERIMENT I. REPEATED INJECTION OF LIOCPODIUM SUSPENSION

1) General case record.

In this experiment six rabbits were used (table 1). None of them died during

the procedure and there were no gross changes of the injected lower limb.

2) Histological findings.

Table 1. Tabulated Summary of the Experiment I.

Number of animal	Weight (g.)	Times of injection	Total amount of injected licopodium (cc.)	Days for which the animal lived after the first injection
1	1900	4	4.0	44
2	1400	2	1.6	19
3	1700	3	3.0	30
4	1900	3	3.0	45
5	2050	3	2.5	28
6	1950	2	3.0	35

Slight proliferation was found of endothelial cells of blood vessels, and nuclei of the endothelial cells were some what pycnotic in many cases. Subendothelial tissue showed a remarkable thickening in some parts, but a moderate thickening in most parts, and vacuolar degeneration and edema were frequently found in it.

In the lumen of medium-sized or small arteries many licopodium particles were found to be completely or almost completely plugged. In the neighborhood of these arteries there were a number of newly formed small blood vessels. In many places about ten particles of licopodium were found to be embolizing an artery, but occasionally only one particle was seen insides a small artery. In some other places from one to several licopodium particles were found to be buried in tunica interna or near tunica media, and less frequently there was a single licopodium in tunica adventitia.

Elastica interna was usually preserved, but sometimes in the licopodiumplugged region of arteries it was apparently broken or disappeared.

Musculature of tunica media was hypertrophied in most cases, and there were no vacuolar degeneration and edema in it.

Tunica adventitia tended to be edematous, and a remarkable perivascular cell infiltration was not present. Around arteries some collections of fibroblast-like cells were found sporadically.

In general dilatation of blood vessels and thickening of vascular wall were more marked in smaller blood vessels.

In arteries which were embolized completely with licopodiums, vascular wall became thinned and the muscular layer was left only partially or disappeared completely and the normal structure of blood vessels was lost

In some cases, e. g. No. 1, there was an organized thrombus in a mediumsized artery and a number of newly formed small vessels were seen in the neighborhood of the thrombus, but not within the thrombus.

Muscle changes of various degrees were seen in proportion to the degree of circulatory disturbance. Thus there was a part in wh ch the muscle did not differ much from normal, or a part where muscle nuclei were more or less increased, or muscle fibers were thinner and smaller and surrounded by the proliferated adipose tissue, so that the structure appears in cross section like a honey-comb. Muscle fibers were granulated or undergoing dissolution. The space among atrophied muscle fibers were filled with fibrous tissue, and sometimes muscle fibers disappeared nearly totally, replaced by the connective tissue. In some places, muscles

became necrotic, the outline of muscle fibers became indistinct, staining lightly with eosin and plenty of fragments of pycnotic muscle nuclei were seen in the interfascicular space. There were many parts in which aggregates of heav ly degenerated muscle bundles were neighbored by those of nearly normal muscle bundles.

Muscle nuclei showed various changes according to the degree of degeneration ; some of them were stained normally by hematoxylin, and others stained lightly, or transparently, or darkly etc.. The shapes of nuclei were round, oval, slender or cylindrical etc.. Occasionally it was difficult to distinguish them from the nuclei of connective tissue cells. Generally speaking, transparent nuclei were large in size and dark ones were small. There were areas where small bleedings were seen in the interstitium of a muscle. In one case muscular multinucleated giant cells were found.

The perineurium of peripheral nerves was edematously swollen and the intima of blood vessels in the perineurium was thickened. Proliferation of nuclei of Schwann's sheath wa, often seen. The changes of nerve fibers were not certain because of the lack of specific stainin .

EXPERIMENT II. REPEATED INJECTION OF A MIXED SUSPENSION OF LICOPODIUM AND HEMOLYTIC STREPTOCOCCUS.

1) General case record.

In this experiment six rabbits were used (table 2). A rabbit (No. 10) died before the end of an observation period. There was no case in which gross changes of the injected lower limb were found.

Table 2. Tabulated Summary of the Experiment II.

Number of animal	Weight (g.)	Times of injection	Total amount of the mixed suspension injected (cc.)	Days for which the animal lived after the first injection
7	1500	2	4	23
8	2400	3	7	25
9	2800	3	7	30
10	2100	1	3	20
11	1900	2	4	35
12	2000	3	6	21

2) Histological findings.

In blood vessels proliferation of the endothelium was slight. In one case a few detached endothelial cells looking like monocytes were seen in the lumen of a blood vessel. Nuclei of endothelial cells tended to be pycnotic. There was not infrequently vacuolization of subendothelial cells of small blood vessels.

The changes of blood vessels in the areas of licopodium embolism were the same as in Experiment I. It was observed that, if licopodium particles obturated the lumen partially, the wall became thinned on the side on which the particles were attached and thickened on the opposite side. Perivascular collection of fibroblast-like cells was sporadically seen and in a few cases there were remarkable perivascular infiltrations of lymphocyte-like mononuclear cells.

Changes of muscle tissue were also the same as in Experiment I. However, the degree of changes in Experiment II seemed more marked than in Experiment I. Neither hemolytic streptococcus nor abscess formation was found anywhere.

Fresh red blood cells were often seen in the interstitium of muscles. Among normal muscle bundles there were degenerated ones distinctly separated from the former. Muscular multinucleated giant cells were occasionally found near the marginal zone of degenerated muscles.

Peripheral nerves were somewhat edematous.

EXPERIMENT III. REPEATED INJECTION OF A SILVER PARTICLE SUSPENSION.

(1) General case record.

In this experiment nine rabbits were used (table 3). Seven died before the end of an observation period (No. 14, 15, 16, 17, 18, 19, 20). Grossly in the injected lower limb there were depilation (No. 19, 20), small ulcer of toes (No. 19), inflammatory swelling (No. 16), necrosis and falling-off of the foot from the ankle-joint (No. 17). These healed after a certain period.

Table 3. Tabulated Summary of the Experiment III.

Number of animal	Weight (g.)	Times of injection	Total amount of injected silver suspensio ı (cc.)	Days for which the animal lived after the first injection
13	1800	2	6	10
14	1850	1	2	14
15	1750	2	3	20
16	2500	2	4	37
17	1900	3	5	135
18	2300	1	2	56
19	2100	1	2	68
20	1950	2	4	120
21	1800	2	3	210

Histological findings.

(2) In arteries where the lumen was filled with more than one silver partlice, there were severe small round cell infiltrations in the wall of the vessels and around them. Such cell infiltrations were intense especially in the animals which died shortly after the injection, but not present in the long surviving animals. In arteries, of which the lumen was obliterated with one silver particle, the normal structure of the vessel wall was lost ; intima and media completely disappeared as if the silver particle alone exsisted in the connective tissue, any reactive change being lacking around the particle.

It seems that the silver particle was impacted tightly into a small blood vessel by the pressure of the blood stream and the vessel wall underwent pressure atrophy.

Some proliferation of endothelial cells and pycnosis of nu lei were seen in other arteries.

Thickening of intima was slight. In Case No. 18, the intima of a medium-sized artery was thickened verrucously. In general, intima was edematous. Media was hypertrophied, but not edemato s. In adventitia and its neighborhood at the site of silver particle impaction, there were severe cell infiltrations as mentioned above. In the vicinity of obturated blood ves els the new formation of abundant small blood vessels was always seen, especially remarkable in Case 17, where the foot fell off from the ankle-joint.

In the muscle tissue, degeneration of various degrees was observed in the same way as in the preceding experiments. Besides the findings described there, here

were scattered some muscle fibers which underwent hyaline degeneration or some other muscle fibers, the outlines of which were stained more intensely with hematoxylin.

Around the blood vessels in the interstitium of muscle tissue, there were fresh bleedings and hemosiderin deposits.

In cases of long-surviving rabbits, muscle fibers were thinned and dispersed and in some parts disappeared, being replaced by the connective tissue. The finding corresponds to that of fibrous myositis.

In all cases there were muscular multinucleated giant cells, seemingly plasmodial.

Changes of peripheral nerves were the same as in the preceding experiments.

EXPERIMNT IV. INTRAMUSCULAR INJECTION OF SILVER PARTICLES

It is questioned whether the changes observed in Experiment III are to be attributed more to the toxicity of silver than to the obstruction of vessels.

In the present experiment, I have carried out the control experiment, in which the same suspension of silver particles as in the previous experiment is injected intramuscularly.

1) General case record.

Two rabbits were used (table 4). 0.4 cc. of the suspension was injected into femoral muscle. During the course of observation after the injection there were no gross changes in the injected part. 10-15 days later the animals were killed and muscle pieces of the injected part were examined histologically.

Table 4. Tabulated Summary of Experiment IV.

Number of animal	Weight (g.)	Times of injection	Total amount of injected suspension (cc.)	Days for which the animal lived after the injection
22	1900	1	0.4	10
23	1750	1	0.4	15

2) Histological findings.

Changes were localized mainly in the injected part. Muscle fibers in the neighborhood of silver particles were atrophic. A number of neutrophil polymorphonuclear leucocytes were present, and among these there were a few eosinophil leucocytes and forign body giant cells. However the changes were not so intense as in Experiment III. In the part where silver particles did not exist, only a small grouping of neutrophil polymorphonuclear leucocytes was seen in the interstitium of muscles. There was no increase of muscle nuclei.

In short, the changes in the muscle tissue were slight and localized for the most part around silver particles. In consideri g the fact that neutrophil polymorphonuclear leucocytes, eosinophil leucocytes and foreign body giant cells were seen, the changes in this experiment seem to be due mainly to the action of silver particles as foreign bodies rather than to the chemical toxicity of silver. Thus it may be assumed that the changes in the previous experiment resulted from the embolism of silver particles.

SUMMARY AND COMMENT

It has been stated that histological changes due to allergic reaction are characteristic, appearing mainly in and around blood vessels. From this view point histological changes in my experiments will be criticized.

My results can be summarized as follows.

In blood vessel there were some proliferation of endothelial cells, pycnosis of their nuclei, (especially verrucous thickening of intima in Experiment III), subendothelial vacuolization, hypertrophy of media, more or less obturation of the lumenby foreign bodies, and perivascular edema. It may not be acceptable that they are allergic changes, because histological criteria for allergic reaction, as Klinge has shown, are fibrinoid swelling of the wall of blood vessels, fibrinous or hyaline thrombus and perivascular infiltration of small round cells, including plasma cells.

Such vascular changes were found in no case of my experiments, while they are quite conspicuous in human thromboangiitis obliterans (Jäger, Wada, Hayashi). Therfore it may be certain that the changes of blood vessels in my experiments are not of allergic nature, but a mere result of circulatory deficit. Thus a tissue damage due to the circulatory arrest does not seem to be allergenic, and therefore it may be assumed that allergic changes in thromboangiitis obliterans are not secondary to a tissue damage, but essentially primary.

In the muscle tissue, there were granular, vacuolar and rarely hyaline degenerations, increase of adipose tissue, regressive changes of muscle fibers nuclei, necrosis of muscle fiber and multinucleated giant cells of plasmodial type. These changes are quite common in usual myositis. Ukawa found them in case of local anaphylaxis of muscle but stated that they were not characteristic of anaphylactic myositis. is no There good reason to believe that these changes are allergic.

In the peripheral nerves, although details were not demonstrable by hematoxylin-eosin staining edema was the main change and some thickening of the intima of blood vessels within the nerves was also seen, allergic changes being absent.

As regards gross changes of the limbs, depilation, small ulcer, and falling-off of the foot from the ankle-joint were observed in Experiment III, but they were never so incurable as those in thromboangiitis obliterans, always healing in a comparatively short period of time. Thus it seems that the disturbance of circulation produced in my experiments can easily be compensated by the formation of collateral channels. Therefore in a persisting circulatory failure as in thromboangiitis obliterans, there must be an ever progressing vascular obturation. This is the very characteristic of the disease and never to be reproduced in experiments.

Since the time when Klinge and others advanced the streptococcus allergy theory, Criep, Mayer, Murphy and Swift etc. succeeded experimentally in the reproduction of a series of allergic histological changes, e.g. rheumatic myocarditis or rheumatic nodular giant cells. In consideration of these facts, I have attempted

to inject intraarterially the hemolytic streptococcus together with licopodiums (Expeirment II), but only a little severer change took place both in blood vessels and muscles as compared to the control (Experiment I), allergic changes being likewise absent.

As to the question whether a severe acute reaction observed in the tributary of the artery, into which silver particles were injected, may be the result of the toxicity of silver, the answer is negative in considering the finding in Experiment IV. The reaction is to be attributed to some mechanical property of silver particles more fitted for embolism.

CONCLUSION

1) By the repeated injection of suspension of licopodium or of silver particle into the femoral artery, definite changes took place in blood vessels, muscles, and nerves of the injected lower limb, but they were not to be regarded as allergic changes.

2) Intensity of histological changes by the injection of silver particles was more marked than that by the injection of licopdium.

3) Even in case of the repeated injection of hemolytic streptococcus together with licopodium, typical allergic changes did not occur.

4) There was one case in which the foot on the injected side fell off from the ankle-joint as the result of necrosis, but the wound healed in a relatively short time. An incurabl ulceration as seen in thromboangiitis obliterans could not be reproduced in my experiments.

5) So far as my experiments are concerned, it seems improbable, even if not impossible, that the devitalized tissue in the body may be allergenic. Thus the allergic changes in thromboangiitis obliterans may well be assumed to be primary and not secondary to the tissue damage as a result of the circulatory failure.

REFERENCES

1) Denecke, K. : Pathologisch-anatomische und klinische Untersuchungen zur Aetiologie der juvenilen Gangrän, Arch. f. kl. Chir., **177**, 821, 1933. 2) Jäger, E. : Zur pathologischen Anatomie der Thromboangiitis obliterans bei juveniler Extremitätengangrän, I Mitteilung, Virch. Arch. f. path. Anat., **284**, 526, 1932, und II Mitteilung, ebenda, **284**, 584, 1932. 3) Klinge, F. : Das Gewebsbild des fieberhaften Reumatismus, Virch. Arch. f. path. Anat., **286**, 344, 1932. 4) Pagel, W. : Pathologie und Histologie der allergischen Erscheinugen, Fortschritte der Allergielehre, von Kallos, P. Karyger, S. Basel, New York. 1939. 5) Ratschow, M. : Beitrag zur Ursache der Extremitätengangrän, Zbl. f. d. Chir., **60**, 821, 1933. 6) Rössle, R. : Ueber den Formenkreis der rheumatischen Gefässveränderungen mit besonderer Berücksichtigung der rheuamtischen Gefässentzündung, Virh. Arch. f. path. Anat., **288**, 780, 1933. 7) Sulzberger, M. B. : Recent immunologic studies in hypersensitivity to tobacco, J.A.M.A. **102**, 11, 1934. 8) Eda, S. : Patho-histological studie on blood vessels of spontaneous gangrene, J. Jap. Surg. Soc. **42**, 937, 1941. 9) Hatano, S. . A contribution to the pathology of nuclei of muscle, Tokyo-Igakukai-Zassi, **39**, 309, 1925, 10) Hayasi, H. and Wada, T. : Histo-pathological changes in spontaneous gangrene, Tr. Soc. Path. Jap. **38**, Editio regionalis, 23, 1949. 11) Karatu, E. : Studies on socalled spontaneous gangrene, J. Jap. Surg. Soc.,**36**, 1389, 1935. 12) Okubo, S. Studies on experimental thrombosis, Kaigun-Guniikai-Zassi, **22** : 80, 1933, and ebenda **22**, 550, 1933. 13) Sato, T. : Studies on spontaneous gangrene, J.Jap. Surg. Soc., **42**, 577, 1941. 14) Suzue, K. and Ueno, M. : The death by sepsis following the amputation of lower limb of

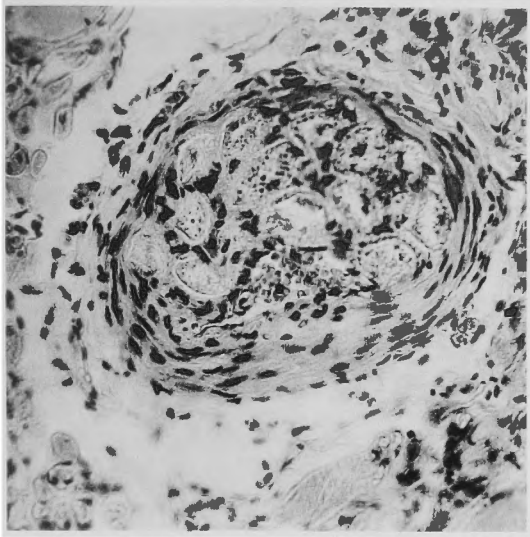

Fig. 1

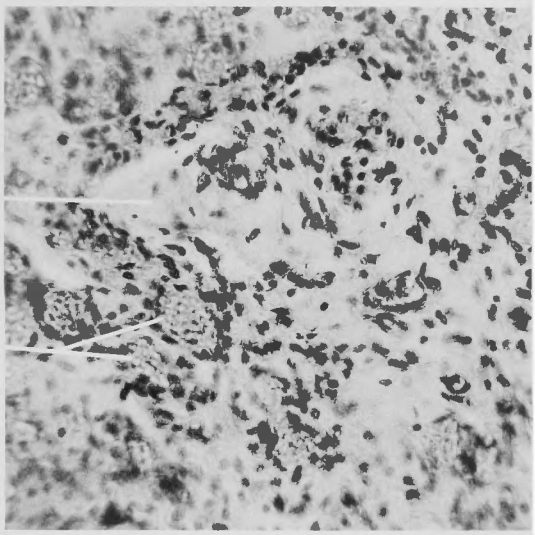

Fig. 4

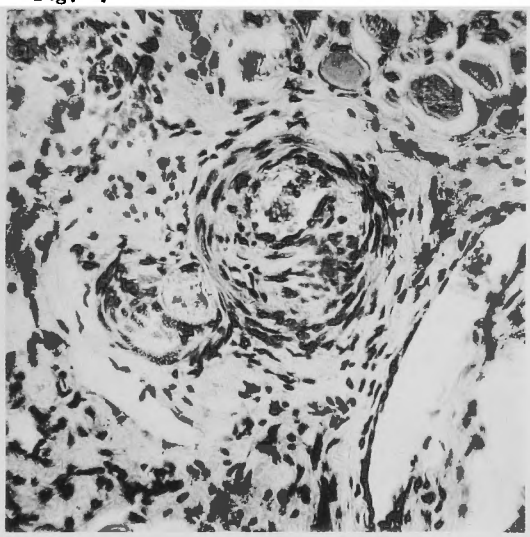

Fig. 2

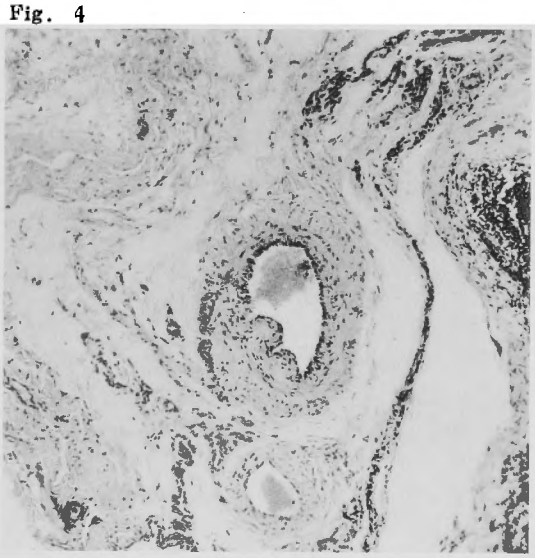

Fig. 5

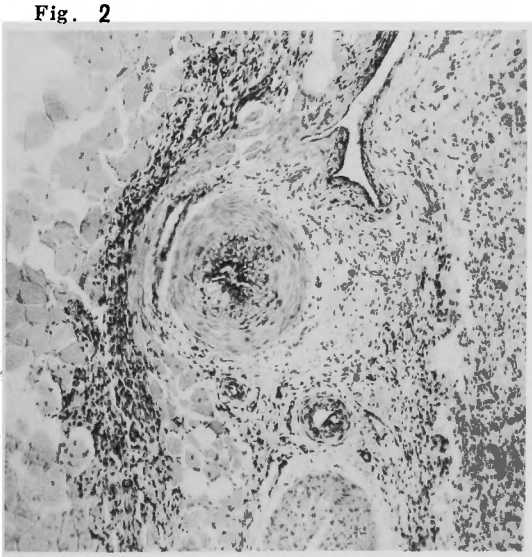

Fig. 3

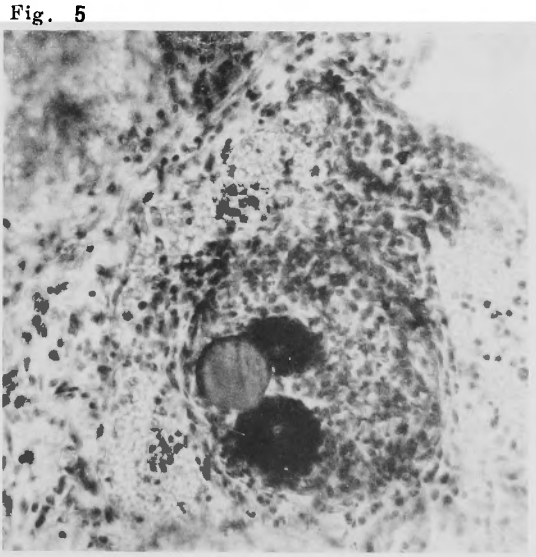

Fig. 6

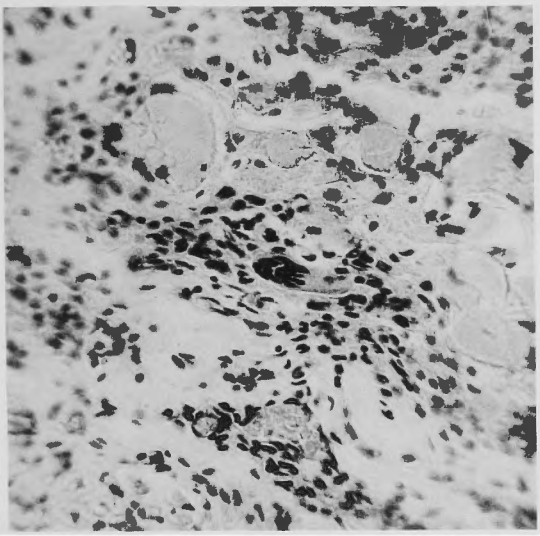

Fig. 7

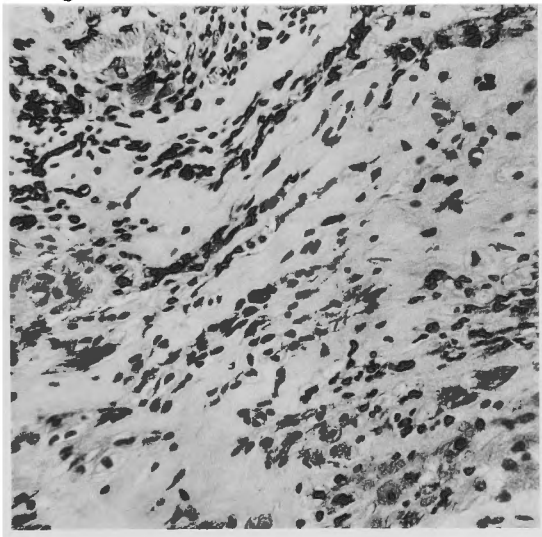

Fig. 8

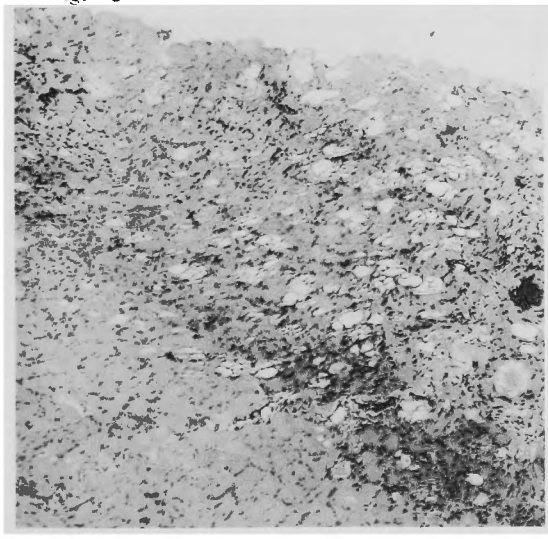

Fig. 9

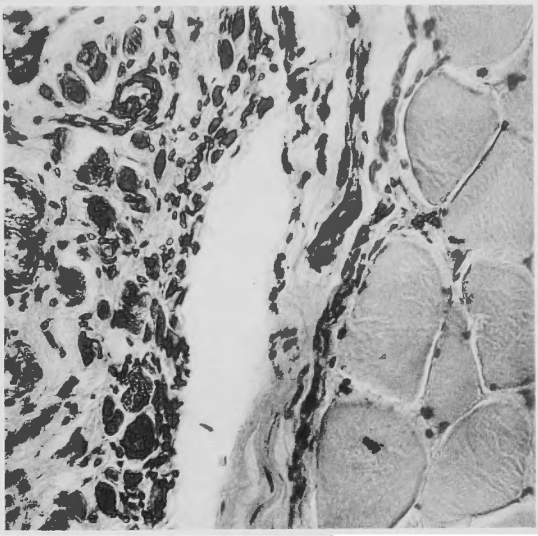

Fig. 10

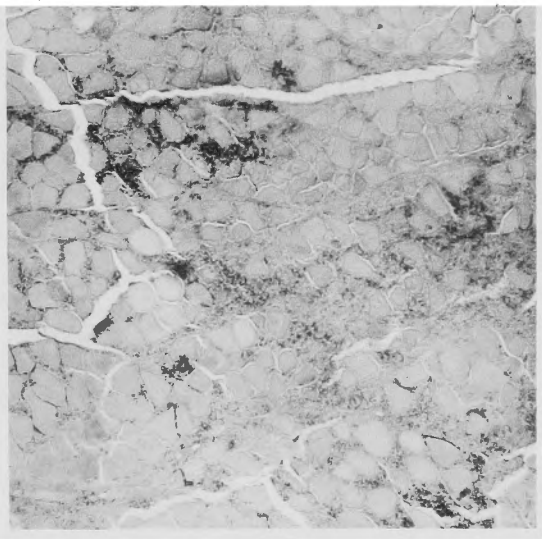

Fig. 11

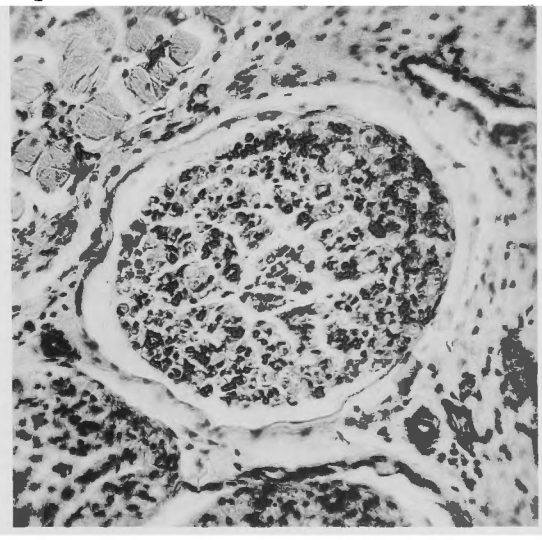

Fig. 12

spontaneous gangrene, Syuzyutu, **2**, 304, 1948. 15) Suzue, K. and Hayasi, H.: A new theory of rheumatism, Nanzando, Tokyo, 1951. 16) Ukawa, Y.: Patho-histological studies on local anaphylaxis, II chapter, The local anaphylaxis in muscle tissue, Kurasiki-tyuobyoin-nenpo, **2**, 13, 1927. 17) Wada, H.: Concerning allergic origin of spontaneous gangrene, Jap. Circul. J., **14**, 265, 1951.

EXPLANATION OF PLATES

Fig. 1. The lumen of an artery is obliterated completely with a number of particles of licopodium. Perivascular edema. ×360

Fig. 2. A licopodium in adventitia ×360

Fig. 3. Proliferation of intima in a small artery. There are three particles of licopodium in the intima. The lumen of the vessel obliterated nearly completely. ×80

Fig. 4. Around obstructed arteries there are newly formed small blood vessels in abundance. A-obstructed and organized artery B-newly formed blood vessels. ×360

Fig. 5. Verrucous thickening of intima of a medium-sized artery. ×80

Fig. 6. The wall of an artery, which is embolized with three silver particles, is heavily infiltrated by leucocytes. ×360

Fig. 7. A muscular multinucleated giant cell which seems to be formed plasmodially. In the center of the photograph. ×360

Fig. 8. Fibrosed muscle tissue. Atrophied muscle fibers are seen on both sides. ×360

Fig. 9. Increase of adipose tissue within a muscle. ×80

Fig. 10. Remarkable difference in histological changes between the two neighboring groups of muscle bundles. Right side is almost normal and left degenerated. ×360

Fig. 11. Necrosis of muscle tissue. ×80

Fig. 12. Peripheral nerves. There seems to be edema beneath the perineurium. ×225

実験的に多数の小栓塞を起した下肢の組織学的変化
——閉塞性血管炎のアレルギー性成因補遺

京都大学医学部外科学教室第一講座（荒木千里教授 指導）
医学士 小 西 誠 三

特発性脱疽の組織学的研究に於て見られるアレルギー性変化に就ては，それがアレルギーに依る一次的変化であるか，又は血行障害の結果起る組織崩壊産物に依る二次的アレルギー変化であるか明でない．この点を明にするため，石松子，銀粒子，溶血性連鎖状球菌等を家兎股動脈に注入し，その下肢に多数の小栓塞をつくつた後組織学的検索を行い，次の結論を得た．

1. 石松子，銀粒子注入に依り，注入下肢の血管，筋肉，神経に高度の変性を認めたがアレルギー性組織変化とは考えられない．

2. 銀粒子注入例の組織変化は石松子注入例より高度である．

3. 溶血性連鎖状球菌を石松子と共に注入したが著明なるアレルギー性組織変化は起らない．

4. 注入側下肢足関節以下の壊死脱落を来した例があつたが，創面は比較的短時日に治癒した．

5. 局所の組織崩壊産物等に依つてはアレルギー性組織変化は仲々起らない．即ち，特発性脱疽に見られるアレルギー性組織変化はアレルギー素因に依る一次的な変化であると思われる．